Predict the Progression of Cervical Intraepithelial Neoplasia by a Novel Marker Folate Combine with FRα, p16 and Ki-67

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Research Article

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Abstract

Background: To study the expression of serum folate and red blood cell (RBC) folate, folate receptor α (FRα), $p^{16INK4a}$ (p16), and Ki-67 at different levels of cervical intraepithelial neoplasia (CIN) and then analyze their role in the progression of CIN and their value as an early warning indicator of CIN progression.

Methods: We randomly collected cervical tissues from patients at the Department of Gynecology in Guangxi Medical University Affiliated Cancer Hospital: Normal controls (149 cases), CIN1 (150 cases), CIN2 (100 cases), and CIN3 (101 cases). The expression of serum folate and RBC folate was detected by the chemiluminescence method, while the expression of FRα, p16, and Ki-67 was detected by Streptavidin-Perosidase (SP) immunohistochemistry.

Results: There was no statistically significant difference in serum folate levels between different grades of CIN (P=0.784), but the RBC folate levels were statistically significant (P=0.015), and there was a negative correlation between RBC folate levels and CIN lesion grades (P<0.05). The FRα, p16, and Ki-67 levels in the CIN group were significantly different from those in the normal control group (P <0.01), and a positive correlation was found (P <0.01); FRα positivity (P=0.000), Ki-67 positivity (P=0.000), and low-level RBC folate (P=0.000) were independent risk factors for the progression of CIN; these indicators were combined to establish a random forest (RF) model in which the Ki-67+FRα model was used as the early warning model of CIN progression.

Conclusions: RBC folate, FRα, p16, and Ki-67 can be used as valuable clinical test indicators for predicting the progression of CIN; the combined detection model of Ki-67+FRα can be used as an early warning model for predicting the progression of CIN.

1 Objects And Methods

1.1 Research object

We randomly collected cervical tissues from patients at the Department of Gynecology in Guangxi Medical University Affiliated Cancer Hospital: normal control (149 cases), CIN1 (150 cases), CIN2 (100 cases), and CIN3 (101 cases). Patients with abnormal ThinPrep cytologic test (TCT) results were further diagnosed using colposcopy and histopathological examination. Patients with nutritional megaloblastic anemia, hemolytic disease, leukemia, localized enteritis, liver disease, and other tumors as well as B vitamin users within 3 months were excluded. All research subjects entered the study with informed consent.

1.2 Methods

1.2.1 Serum and RBC folate concentration detection

We collected 5 ml of fasting cubital venous blood from the subjects and placed it in a nonanticoagulant tube, left it at room temperature for 3 hours, centrifuged it for 15 minutes (3000 rpm), carefully collected the supernatant, and stored it in an ultralow temperature refrigerator (-80°C) for batch testing. The sample could be kept in a frozen state for no more than 6 months before testing. The chemiluminescence method was used to determine serum folate and RBC folate levels. Instruments and reagents, including the folate assay kit, chemiluminescence substrate Lumi-Phos*530, Access Wash Buffer, folate calibrator, and automatic luminescence analyzer, were purchased from Beckman Coulter, USA.

1.2.2. Detection of FRα, P16, Ki-67

The pathological HE staining slices and their corresponding tissue wax blocks for all patients in the group were selected, and the corresponding tissue wax blocks were verified and checked for integrity, which could be used for immunohistochemistry experiments. After the wax blocks were cut into thin slices for dewaxing and dehydration, the

endogenous enzymes in them were removed, the antibodies were dripped in after high-pressure antigen restoration; then, hematoxylin was used for counterstaining to make immunogrouped slices. Among those, the antibody used for FRα detection was mouse anti-human FRα monoclonal antibody (ab3361) purchased from Abcam Company; the antibody used for Ki-67 detection was mouse anti-human Ki-67 monoclonal antibody (clone number: MIB-1), purchased from Fuzhou Maixin Reagent Company. In addition, the antibody used for p16 detection was mouse anti-human $P^{16INK4a}$ monoclonal antibody (clone number: 3G5D5), which was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, where the ready-to-use immunohistochemical hypersensitivity SP kit and DAB staining kit used for P16 detection were also purchased.

FR-α immunohistochemistry is positively expressed on the cervical epithelial cell membrane, and the positive effect product is brown–yellow particles. FR-α scoring standard: according to the comprehensive score of dyeing degree and dyeing area: the dyeing degrees for no coloring, light brown, brown, and dark brown were, respectively, 0 points, 1 point, 2 points, and 3 points; stained areas of 0, 1–25%, 26–50%, and greater than 50% were, respectively, 0, 1, 2 and 3. The two scores were added together, and 0 to 1 was considered negative (-), 2 points were rated as positive (+), 3 to 4 points were rated as weakly positive (++), and 5 to 6 points were rated as strongly positive (+++).

The results of p16 and Ki-67 were judged by using the semiquantitative method,which was in according with the proportion of positive cells in the vertical distribution of the cervical scaly epithelial. The positive marker of basal cells was marked as 1+, the lower 1/3 of the squamous epithelium was marked as 2+, the lower 2/3 was marked as 3+, and the lower 2/3 was more than the lower 2/3 to the full thickness. The coloring was marked as 4+.

1.3 Statistical analysis

SPSS 25.0 software was used to establish a database. Nonnormal quantitative data are described by the median ± interquartile range ($M \pm Q$), and the Kruskal–Wallis H test and Spearman rank correlation were used for analysis. The rank-sum test and the rank correlation test were used for the immunohistochemical staining results of FRα, p16, and Ki-67. $P < 0.05$ was considered statistically significant. Meaningful test indicators of the above research were analyzed by univariate analysis and multivariate logistic analysis. Based on the above results, different combinations of significant factors were selected to establish random forest models.

2 Results

2.1 Serum folate and RBC folate in patients with CIN

2.1.1 Serum folate test results

The serum folate level in the normal control group was 17.08 ± 13.66 nmol/L; the serum folate levels in the CIN1, CIN2, and CIN3 groups were 16.56 ± 12.95 nmol/L, 16.81 ± 12.40 nmol/L, and 16.52 ± 12.91 nmol/L, respectively. The Kruskal–Wallis H test revealed that the overall distribution of serum folate levels in the four groups was not statistically significant ($H = 1.07$, $P = 0.784$) (Table 1).

Table 1
The relationship between serum folate and CIN

Group	Serum folate(nmol/L)	*H*	*P*
Normal	17.08 ± 13.66	1.07	0.784
CIN1	16.56 ± 12.95		
CIN2	16.81 ± 12.40		
CIN3	16.52 ± 12.91		

2.1.2 RBC folate test results

The RBC folate of the normal control group was 7.37 ± 5.67 nmol/L; the RBC folate levels of CIN1, 2, and 3 groups were 6.59 ± 5.16 nmol/L, 6.41 ± 4.82 nmol/L, and 6.44 ± 4.58 nmol/L, respectively. Kruskal–Wallis H test indicated that the overall distribution of RBC folate levels in the four groups was statistically significant ($H = 10.469$, $P = 0.015$) (Table 2). Furthermore, the 50% value of the RBC folate content of the normal control group was used as the boundary, and ≤ 7.37 nmol/L was the low folate level for analysis. The results verified that the low folate rates were higher in the CIN 1, 2, and 3 groups than in the control group. The Mentel Haenszel chi-square test demonstrated that there was a linear relationship between the RBC folate level and the grade of CIN lesions, $\chi^2 = 5.901$, $P = 0.015 < 0.05$. The Pearson correlation results confirmed that there was a negative correlation between RBC folate and the grade of CIN lesions, $R = 0.109$, $P = 0.015 < 0.05$. As the severity of CIN lesions worsened, the RBC folate deficiency rate gradually increased, and the RBC folate level gradually decreased.

Table 2. The relationship between RBC folate and CIN

Group	Content(nmol/L)	Rate	*H*	*P*
Normal	7.37 ± 5.67	75(50.3%)	10.469	*0.015*
CIN1	6.59 ± 5.16	90(60.0%)		
CIN2	6.41 ± 4.82	67(67.0%)		
CIN3	6.44 ± 4.58	65(64.4%)		

2.2 FRα, p 16, and Ki-67 immunohistochemistry results

The rank-sum tests of FRα, p16, and Ki-67 staining in the CIN and control groups at each level verified that there were significant differences between each group and the normal control group (CIN1: $H = 260.298$, $P < 0.01$; CIN2: $H = 373.769$, $P < 0.01$; CIN3: $H = 329.886$, $P < 0.01$). The Spearman rank correlation test demonstrated that there was a positive correlation between the positive expression of FRα, P16, and Ki-67 and the grade of CIN lesions (CIN1: $r = 0.684$, $P < 0.01$; CIN2: $r = 0.826$, $P < 0.01$; CIN3: $r = 0.778$, $P < 0.01$), indicating that the positive expression rate of FRα, p16, and Ki-67 gradually increased with an increase in CIN lesion grade (Table 3).

Table 3
The expression of FRα, p16 and Ki-67 in each level of CIN

Group	N	FRα					P16					Ki-67				
		-	+	++	+++	++++	-	+	++	+++	++++	-	+	++	+++	++++
Normal	149	15	133	1	0	0	149	0	0	0	0	0	148	0	1	0
CIN1	150	4	136	10	0	0	120	23	7	0	0	1	116	25	7	1
CIN2	100	2	29	66	3	0	12	7	78	2	1	0	14	3	80	3
CIN3	101	3	15	61	22	0	4	14	11	72	0	0	14	1	12	74

2.3 Logistic regression analysis

To explore the risk factors for the occurrence and progression of CIN, the four indicators FRα, P16, Ki-67, and RBC folate were included in the univariate logistic analysis (a *P* value less than 0.01 was considered to be statistically significant and was included in the multivariable analysis). The multivariable logistic analysis confirmed that FRα positivity (*HR* 9.436, *95% CI* 3.569–24.945, *P* = 0.000), Ki-67 positivity (*HR* 16. 389, *95% CI* 5.203–51.620, *P* = 0.000) and low levels of RBC folate (*HR* 0.917, *95% CI* 0.849–0.989, *P* = 0.000) were independent risk factors for the occurrence and progression of CIN(Table 4).

Table 4
Logistic regression of risk factors for CIN

	Univariate logistic analysis			**Multivariate logistic analysis**		
	HR	***95% CI***	***P***	***HR***	***95% CI***	***P***
FRα	11.867	6.243–22.556	**0.000**	9.436	3.569–24.945	**0.000**
p16	5.991	/	0.990	/	/	/
Ki-67	14.306	5.296–38.641	0.000	16.389	5.203–51.620	**0.000**
RBC folate	0.926	0.876–0.980	0.008	0.917	0.849–0.989	**0.000**

2.4 Establishment of Radom forest models for CIN Progression

According to the results of multivariate logistic regression analysis, different combinations of significant risk factors that promote the progression of CIN were selected to establish random forest models(Table 5). According to the accuracy rate, out-of-bag (*OOB)* error value and Area under the curve of ROC(AUC) of each model. Among the four models of predicting CIN progression, the Ki-67 + FRα model had the highest accuracy rate and the smallest *OOB* error value, while the *AUC* was relatively ideal(Fig. 1). For this reason, it can be used as a candidate for an early warning model for predicting the progression of CIN.

Table 5. Random forest models for predicting CIN progression

NO.	RF models	accuracy	*AUC*	*OOB*
1	RBC folate + Ki-67	70.67%	79.96%	26.29%
2	RBC folate + FRα	72.67%	73.61%	31.71%
3	**Ki-67 + FRα**	**79.33%**	**85.91%**	**25.14%**
4	RBC folate + FRα + Ki-67	76.67%	87.49%	26.29%

3. Discussion

3.1 Folate

Folate is an essential vitamin for humans that participates in the metabolism of the one-carbon unit. The normal level of folate in the body ensures balanced DNA synthesis and DNA methylation. It plays an important role in cell proliferation and is essential for maintaining genome stability. This is due to its two functions: on the one hand, as a methyl donor of deoxythymidine monophosphate to form deoxythymidine triphosphate, which is used for DNA synthesis and repair, and on the other hand, it is synthesized with the common methyl donor S-adenosylmethionine for cytosine methylation. Therefore, a lack of folate can cause genomic DNA strand breaks, chromosomal instability, uracil mismatches, etc., disrupt DNA synthesis and repair, and affect nucleic acid and histone methylation modifications, thus increasing the risk of tumors[3].

In the occurrence of cervical cancer, most scholars believe that high-risk HPV is not randomly inserted into the host genome but often integrates into the unstable and transcriptionally active regions of the cervical epithelial cell genome and inhibits its function. This integration is most likely to occur in the fragile histidine triad (FHIT) gene at the chromosomal fragile site, FRA3B. The FHIT gene is a special tumor suppressor gene, and its abnormal expression is closely related to the occurrence and development of cervical cancer. FHIT gene central pattern generator (CPG) methylation is one of the important mechanisms leading to its functional inactivation. As folate is directly involved in DNA methylation as a methyl donor in the body, its deficiency may affect chromosomal stability at the FRA3B site located in the FHIT gene, which is prone to forming gaps or breaks on metaphase chromosomes, thereby increasing the risk of high-risk HPV virus infection and leading to cancer of the cervix[4]

This study revealed that the overall distribution of serum folate levels in different pathological groups was not statistically significant, while in recent years, many studies have indicated that low serum folate is related to CIN[5]. Considering that serum folate only reflects the early changes in folate levels in the body that are affected by dietary and absorption factors, the possibility of error is not ruled out. On the other hand, RBC folate ought to represent the storage levels of folate in the body, which are relatively stable. Therefore, this study also analyzed the relationship between RBC folate and CIN simultaneously. After analysis, it was found that there was a negative correlation between RBC folate and the grade of CIN lesions. As the degree of CIN lesions worsened, the RBC folate deficiency rate gradually increased, and the RBC folate level gradually decreased. Univariate logistic analysis and multivariable logistic analysis were used to further determine that low RBC folate levels were an independent risk factor for the progression of CIN. In addition, related studies have pointed out that a high folate state can inhibit the possibility of viral nucleic acid integration into host cells. Appropriate folate supplementation has a protective effect on CIN. Folate supplementation has effects on cervical precancerous lesions, which may provide new strategies for cancer prevention and treatment[6].

In this study, the 50% value of the normal control group was used to divide the RBC folate level. However, whether there is a more appropriate delimitation point to improve the accuracy of the RBC folate level in detecting diseases requires further analysis of a large amount of data. On the other hand, as this study failed to find a relationship between serum folate and CIN, the absorption, metabolism, and transportation of folate in the diet may be affected by various factors and should be further explored.

3.2 FRα

The immortal proliferation of malignant tumor cells requires much higher than normal levels of purines and pyrimidines. Therefore, more folate needs to be taken into the cell. Folate transport mediated by high-affinity folate receptors is the main mechanism of folate ingestion by malignant tumor cells[7].

Folate receptors are synthesized by the Id protein gene family of chromosome 11 q13.3, which include primarily four subtypes of FRα, FRβ, FRδ, and FRγ. FRα is an antigen protein that is specifically and highly expressed by tumor cells. It binds to folic acid with high affinity, causing them to be endocytosed and taken into cells. FRα expression is low in nonmalignant tumor tissues but overexpressed on the surface of a variety of solid tumor cells, which is closely related to the occurrence and progression of tumors[8]. Research has uncovered that FRα is an important activator of the extracellular signal-regulated kinase (ERK) pathway. Its high expression in CIN and cervical cancer could activate the ERK pathway, upregulate the expression of proto-oncogenes, and promote tumor cell proliferation and apoptosis. Downregulating the expression of FRα could reduce the uptake of folate in cells, affect DNA synthesis, break chromosomes, and arrest the cell cycle in the G0/G1 phase, thereby inhibiting the proliferation of tumor cells[9]. This study also found that there were significant differences in the staining of RFα between the CIN group of each grade and the normal control group, the positive expression rate increased with an increase in the disease grade, and there was a positive correlation with the disease grade. Univariate logistic analysis and multivariable logistic analysis revealed that FRα positivity is an independent risk factor that promotes the progression of CIN.

In recent years, some researchers have developed folate receptor-mediated cervical dyeing (FRD) analysis to detect cervical cancer. When staining the cervix with FRD, the compounds of folate–deoxidized methylene blue specifically bound to the receptors on the tumor cells, and the deoxidized methylene blue was oxidized, thus changing the original brown color of the FRD and coloring the cotton swab. This study emphasized that FRD had higher sensitivity and higher specificity in screening high-grade CIN, which was higher than that of TCT and HPV. In addition, as FRD is cost-effective, it is convenient and quick to operate. Moreover, the results of FRD tests can be easily identified and can be widely used in places where there is a lack of colposcopy services and histopathology rooms[10].

3.3 p16

P16 is a cyclin-dependent protein kinase inhibitor (CDKI) that is encoded by the CDKN2A2655 gene located on the short arm of chromosome 9 (9p21.3). Its production P16 protein can interact with cyclin. D competitively binds to cyclin-dependent protein kinase 4 (CDK4) or cyclin-dependent protein kinase 6 (CDK6) and specifically inhibits the activity of CDK4 or CDK6, making it unable to phosphorylate retinoblastoma protein (pRb), thereby preventing cells from entering the S phase from G1 phase and ceasing the development of the cell cycle[11]. In cervical cells infected with HPV, persistent infection of the cervix can cause irreversible changes, leading to carcinoma in situ and ultimately to invasive cervical cancer. The integration of HPV DNA into the host genome can induce the expression of E6 and E7. The E7 protein competes with the cell cycle control protein pRb and induces the overexpression and accumulation of p16 in cells through a negative feedback loop by interfering with the pRb-E2F1 pathway. Therefore, p16 is considered a surrogate marker for persistent high-risk HPV infection. The overexpression of p16 has been observed in most cervical cancers and cancers[12]. This study verified that there were significant differences in p16 staining between the CIN group of each grade and the normal control group, that the positive expression rate increased with increasing disease grade, and that there was a positive correlation with disease grade. However, univariate logistic analysis did not verify that p16 is an independent risk factor for CIN progression, which is inconsistent with the conclusions of other studies. At present, errors are not ruled out, and more in-depth large sample studies are needed for further verification.

3.4 Ki-67

Ki-67 is a cell proliferation marker, a nuclear nonhistone protein encoded by the MKI-67 gene, and is expressed in all stages of the cell cycle, except for the G0 segment, which plays a variety of functions in regulating cell cycle progression. During mitosis, Ki-67 participates in the formation of the chromosome perimeter, which acts as a protective sheath around the chromosome and provides a platform during the nucleolar assembly process. Ki-67 is used as a biological surfactant that can prevent the aggregation of chromosomes after mitosis and the disassembly of the nuclear envelope[13]. Therefore, as a marker of cell proliferation, Ki-67 can predict the malignant potential of tumors. This study found that there were significant differences in the staining of Ki-67 between the CIN group of each grade and the normal control

group, the positive expression rate increased with the increase of the disease grade, and there was a positive correlation with disease grade. Univariate logistic analysis and multivariate logistic analysis revealed that Ki-67 positivity is an independent risk factor that promotes the progression of CIN.

Liu et al.[14]demonstrated that the p16 test alone revealed good sensitivity, while the Ki-67 test alone showed good specificity. Combining the two tests, double positivity can be used to diagnose or monitor women receiving high-level CIN/VAIN treatment. P16/Ki-67 dual-staining cytology is of great significance in the screening and trialing of cervical cancer and precancerous lesions. It provides a good risk marker for the stratification of HPV-positive women, including normal cytological patients, and the identification of high-grade CIN from women diagnosed with ASCUS or LSIL. Compared with Pap cytology and HPV detection, it has a higher sensitivity and specificity in detecting cervical precancerous lesions and cervical cancer. However, whether p16/Ki-67 dual-staining cytology can be a method of diagnosing and following up CIN progression may require a longer follow-up time for further clinical studies.

3.5 The predictive models of CIN

In recent years, many prediction models of relevant CIN progress have been established from different perspectives. This study showed from the establishment of random forest models of tumor-related detection indicators that the Ki-67 + FRα model could be used as a candidate for an early warning model for predicting CIN progress. Through the analysis of clinical factors related to the occurrence and progression of CIN and the core genes located in the common pathways related to CIN.,Chen et al.[15]reported that the factors related to the progression of CIN, included older age, premenopause, and multiple parties, the significant genes of TGFBR2, FOXO1, CSKN1A1, PRKCI, and CTBP2, and the factors related to the occurrence of CIN. including HPV infection, TCT result diagnosis of CINII+, and the significant genes of TGFBR2, FOXO1, and CTBP2. Based on the above results, different combinations of significant clinical factors and genes were selected to establish random forest models. As a result, a model (TGFBR2 + CSKN1A1 + PRKCI + FOXO1 + CTBP2 + premenopause + multiple parity + older age) was selected as the model for CIN progression. Likely, the model (CTBP2 + FOXO1、HPV infection,、TCT result diagnosed of CINII+) was considered the model of CIN occurrence. On the other hand, Zhou et al.[16]established a regression model to predict cervical squamous cell carcinoma with a backward logistic stepwise regression method and found that the combined test of hTERC gene amplification, HR-HPV viral load, and MCM5 protein could be used for prediction and evaluation of cervical lesions. The establishment of these early warning models has continuously improved the CIN detection system.

4. Conclusions

In summary, we selected the detection indicators of folate, FRα, P16, and Ki-67, which have been confirmed to be related to the occurrence and development of tumors. In this study, FRα positive, Ki-67 positive, and low-level RBC folate are independent risk factors for the progression of CIN. For patients with Ki-67 and FRα positive, it is necessary to be vigilant regarding the occurrence and development of CIN.

Abbreviations

Full names	Abbreviation
red blood cell	RBC
folate receptor α	FRα
p16INK4a	p16
cervical intraepithelial neoplasia	CIN
Streptavidin-Perosidase	SP
random forest	RF
human papilloma virus	HPV
ThinPrep cytologic test	TCT
fragile histidine triad	FHIT
central pattern generator	CPG
extracellular signal-regulated kinase	ERK
folate receptor-mediated cervical dyeing	FRD
cyclin-dependent protein kinase inhibitor	CDKI
cyclin-dependent protein kinase 4	CDK4
cyclin-dependent protein kinase 6	CDK6
phosphorylate retinoblastoma protein	pRb
out-of-bag	OOB
Area under the curve of ROC	AUC
receiver operating characteristic curve	ROC

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were approved by the Ethics Committee of Guangxi Medical University affiliated Cancer Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

He Wang: Project development, Manuscript writing

Tingting Liu: Manuscript writing, Statistical analysis

Mengjie Chen: Statistical analysis

Xueqin Li: Data collection

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Figures

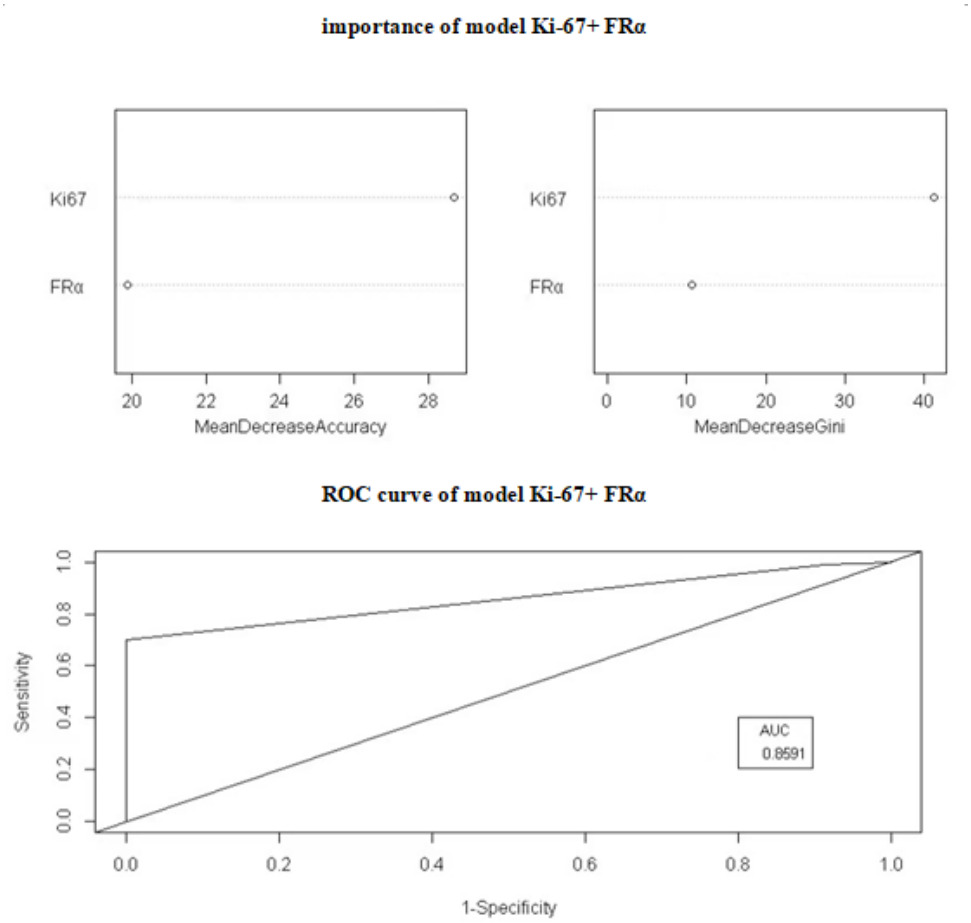

Figure 1

the weight of factors in model Ki-67+ FRα. the ROC curve of model Ki-67+ FRα.